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LIPOLYTIC ENZYME VARIANTS HAVING PHOSPHOLIPASE ACTIVITY

FIELD OF THE INVENTION

The present invention relates to a variant of a parent lipolytic enzyme, and more specifically to such a variant having higher phospholipase activity than the parent lipolytic enzyme. The invention also relates to a DNA sequence encoding the variant, a vector comprising the DNA sequence, a transformed host cell harboring the DNA sequence or the vector, to a method of producing the variant, and to methods of using the variant.

BACKGROUND OF THE INVENTION

Phospholipases are useful in a variety of industrial applications, e.g. in baking and treatment of vegetable oil to reduce the content of phospholipid.

Phospholipases are known from a number of biological sources, including animal sources and microorganisms.

F. Hara et al., JAOCS, 74 (9), 1129-32 (1997) indicates that some lipases have a certain phospholipase activity, whereas most lipases have little or no activity on phospholipids. Thus, phospholipase activity has been described in the lipases from guinea pig pancreas, *Fusarium oxysporum* and *Staphylococcus hyicus*, and attempts have been made to relate the phospholipase activity to the structure of the lipase. WO 98/26057; M.D. van Kampen et al., Chemistry and Physics of Lipids, 93 (1998), 39-45; A. Hjorth et al., Biochemistry 1993, 32, 4702-4707.

SUMMARY OF THE INVENTION

The inventors have found that starting from a given parent lipolytic enzyme, it is possible to make variants with a higher phospholipase activity by modifying the amino acid sequence in the vicinity of the C-terminal and the alcohol binding site.

Accordingly, the invention provides a variant of a parent lipolytic enzyme, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue, and

b) has a higher phospholipase activity than the parent lipolytic enzyme.

The alteration may be at a position which is within 10 amino acid positions from the C-terminal of the mature protein or corresponds to such a position in the *H. lanuginosa* lipase. The parent lipolytic enzyme may particularly be one having a lid and an alcohol binding site, and the alteration may alternatively be at a position which is

i) no more than 10 Å from the C atom at the sn2 position of the glycerol part of a substrate triglyceride, or

ii) in the lipolytic enzyme lid.

The invention also provides a DNA sequence encoding the variant, an expression vector comprising the DNA sequence, a transformed host cell harboring the DNA sequence or the expression vector, and to a method of producing the variant by cultivating the transformed host cell so as to produce the variant and recovering the variant from the resulting broth. Further, the invention provides processes using the variant in baking or degumming of vegetable oil.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows an alignment of lipase sequences.

Figs. 2-3 show firmness and elasticity, respectively, of bread. Details are described in the Examples.

DETAILED DESCRIPTION OF THE INVENTION

Parent lipolytic Enzyme

The lipolytic enzyme to be used in the present invention is one that can hydrolyze ester bonds. Such enzymes include, for example, lipases, such as triacylglycerol lipase (EC 3.1.1.3), lipoprotein lipase (EC 3.1.1.34), monoglyceride lipase (EC 3.1.1.23), lysophospholipase, ferulic acid esterase and esterase (EC 3.1.1.1, EC 3.1.1.2). The numbers in parentheses are the systematic numbers assigned by the Enzyme Commission of the International Union of Biochemistry in accordance with the type of the enzymatic reactivity of the enzyme.

The parent lipolytic enzyme may have a lid and an alcohol binding site, e.g. a fungal lipolytic enzyme. Examples of such parent lipolytic enzymes are lipolytic enzymes of the *Humicola* family and the *Zygomycetes* family.

The *Humicola* family of lipolytic enzymes consists of the lipase from *H. lanuginosa* strain DSM 4109 and lipases having more than 50 % homology with said lipase. The lipase from *H. lanuginosa* (synonym *Thermomyces lanuginosus*) is described in EP 258 068 and EP 305 216; it has an amino acid sequence of 269 amino acids as shown in Fig. 5a-5b of EP 305 216; it is also referred to as Lipolase(R).

The *Humicola* family also includes the following lipolytic enzymes (with publication or access number of the amino acid sequence in the EMBL, GenBank or GeneSeqn databases in parentheses): *Aspergillus foetidus* lysophospholipase (T88341), *A. oryzae* lipase PLA1 (V31126), *Penicillium camembertii* lipase (E04336), *Aspergillus*

oryzae lipase ((D85895), *Fusarium oxysporum* lipase/phospholipase (EP 130064, WO 98/26057), *Fusarium heterosporum* lipase (T09086), *A. niger* lipase/ferulic acid esterase (Y09330), *Aspergillus tubingensis* lipase/ferulic acid esterase (Y09331).

The *Zygomycetes* family comprises lipases having at least 50 % homology with the lipase of *Rhizomucor miehei* (P19515). This family also includes the lipases from *Absidia reflexa*, *A. sporophora*, *A. corymbifera*, *A. blakesleeana*, *A. griseola* (all described in WO 96/13578 and WO 97/27276) and *Rhizopus oryzae* (previously *R. niveus*, *R. delemar*, *R. japonicus*, P21811).

It is of particular interest to derive a variant with phospholipase activity from a parent lipolytic enzyme having no or very little phospholipase activity, e.g. corresponding to a ratio of phospholipase activity to lipase activity below 0.1 PHLU/LU or below 50 PHLU/mg.

Lipolytic enzyme variants

The lipolytic enzyme variant of the invention comprises an alteration (i.e., an insertion, a deletion or a substitution of an amino acid residue), at a position in one of the following overlapping regions:

i) near the C-terminal, i.e. within 10 amino acid positions from the C-terminal or corresponding to such a position in the *H. lanuginosa* lipase, or

ii) at the "alcohol binding site", i.e. no more than 10 Å from the C atom at the sn2 position of the glycerol part of a substrate triglyceride, or

iii) in the lid.

The total number of alterations in the above regions is typically not more than 20, e.g. not more than 10 or not more than 5, and there may be as little as 1 or 2 alterations in the above regions.

In addition, the lipolytic enzyme variant of the invention may optionally include other modifications of the parent enzyme, typically not more than 10, e.g. not more than 5 alterations outside of the above regions. One example is an N-terminal extension as described in WO 97/04079, typically consisting of 1-10 amino acid residues, e.g. AS, SPIRR or E1SPIRPRP.

Alterations near C-terminal

As stated above, the variant of the invention may have one or more alterations within 10 amino acid positions from the C-terminal of the mature protein, or at positions corresponding to such positions in the *H. lanuginosa* lipase, i.e. positions 260-269 of

the *H. lanuginosa* lipase. Corresponding positions may be found by alignment of the two sequences as described later in this specification.

The lipolytic enzyme variant may be truncated by deleting amino acid residues corresponding to the first 1, 2, 3, 4, 5 or 6 positions at the C-terminal. A truncated variant may have improved thermostability.

Alternatively, the variant may carry a peptide extension at the C-terminal and/or the N-terminal. The C-terminal extension may consist of 1-10 amino aicd residues, e.g. A, P, AG, DG, PG, AGG, PVGF, AGRF, PRGF, AGGF or AGGFS; or it may consist of 40-50 residues, e.g., consisting of the 48 C-terminal residues of the *Fusarium oxysporum* lipase AGGFSWRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS. The C-terminal extension may increase the phospholipase activity.

Some alterations in the region overlapping with the alcohol binding site are described below.

A preferred alteration is a substitution at a position corresponding to G266 in the *Humicola lanuginosa* lipase, preferably with an amino acid of intermediate size, e.g. A, C, D, N, L, I, S, T, P or V. Such alteration alone has been found sufficient to increase the phospholipase activity.

Other preferred alterations are such that alter the tertiary structure, e.g. by introducing bulky side chains or by disrupting the bond angles, e.g. by introducing Pro. Such alterations may be made at positions corresponding to positions G263, L264, I265, T267 or L269 in the *Humicola lanuginosa* lipase. Some preferred substitutions are G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; T267A,Q or L269N.

Alteration in lipase lid

As stated above, the amino acid sequence of the parent lipolytic enzyme may be modified in the lid region of the lipase described in Brady et al., Nature 343, 1990, pp. 767-770 and in Brzowski A M et al., Nature, 351: 491 (1991). In the *H. lanuginosa* lipase, the lid is located at positions 80-100, and the modification may particularly be made at positions 82-98.

The variant typically contains no more than 5 alterations in the lid region; it may contain 0, 1, 2 or 3 alterations. A preferred alteration is a substitution of an amino acid corresponding to G91, D96 and/or E99 in the *Humicola lanuginosa* lipase with a neutral or positively charged amino acid, e.g. a substitution corresponding to G91A, D96S,W and/or E99K.

Alteration in alcohol binding site

As already stated, the amino acid sequence of the parent lipolytic enzyme may be modified at a position which is within 10 Å (e.g. within 8 Å, particularly within 6 Å) of

the C atom at the sn2 position of the glycerol part of a substrate triglyceride. This region will be referred to as the "alcohol binding site" of the lipase; it is described in Brzowski A M et al., Nature, 351: 491 (1991); Uppenberg et al., Biochemistry, 1995, 34, 16838-16851; A. Svendsen, Inform, 5(5), 619-623 (1994).

The variant typically contains no more than 10 alterations in the alcohol binding site, e.g. 1, 2, 3, 4, 5 or 6 alterations.

The alteration may particularly be in that part of the alcohol binding site which comes within 20 positions (e.g. within 10 positions) of the C-terminal.

For the *Rhizomucor miehei* lipase, the extent of the alcohol binding site can be found from the PDB file "5tgl.pdb" available in Structural Classification of Proteins (SCOP) on the Internet, at http://pdb.pdb.bnl.gov, showing the complex with the inhibitor n-hexylphosphonate ethyl ester which mimics the substrate. This is described in Brzowski et al. (supra) and Brady et al. (supra). The following amino acid positions lie within 10 Å of the sn2 position in the *Rhizomucor miehei* lipase: 25, 28, 80-84, 88, 143-146, 175, 203, 205, 254-255, 257-259, 264-267. The following are within 8 Å: 81-83, 144, 257-258, 265-267, and the following within 6 Å: 82, 144, 257, 266.

In the *Humicola lanuginosa* lipase, the following positions are within 10 Å of the sn2 position: 18, 21, 81-85, 89, 145-148, 172, 201, 203, 255-256, 258-260, 264-267. The following are within 8 Å: 82-84, 89, 146, 258-259, 265-267, and the following within 6 Å: 83, 146, 258, 266.

Homology and alignment

For purposes of the present invention, the degree of homology may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

In the present invention, corresponding (or homologous) positions in the lipase sequences of *Rhizomucor miehei* (rhimi), *Rhizopus delemar* (rhidl), *Thermomyces lanuginosa* (former; *Humicola lanuginosa*) (SP400), *Penicillium camembertii* (Pcl) and *Fusarium oxysporum* (FoLnp11), are defined by the alignment shown in Figure 1.

To find the homologous positions in lipase sequences not shown in the alignment, the sequence of interest is aligned to the sequences shown in Figure 1. The new sequence is aligned to the present alignment in Fig. 1 by using the GAP alignment to the most homologous sequence found by the GAP program. GAP is provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8,

August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45). The following settings are used for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Preferred variants

Some preferred variants of the *H. lanuginosa* lipase have the following modifications. Alterations given in parentheses are uncertain. FLR numbers are the inventors' reference numbers. Corresponding alterations may be made in other parent lipolytic enzymes.

FLR2: E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

FLR3: E1A, G91A, D96W, E99K, E239C, Q249R, P256A, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G273F, (274S)

FLR4: E1A, G91A, D96W, E99K, N248T, Q249R, W260Q, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

FLR5: SPIRR, G91A, D96W, E99K, W260C, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272, G273F, (274S)

FLR6: SPIRR, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

FLR7: E1A, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

FLR29: E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

FLR30: SPIRR, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

FLR31: SPIRR, G91A, D96W, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

FLR37: E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N

FLR39: E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A

FLR41: E1A, G91A, D96W, E99K, Q249R, G263E, G266D, L269N, 270P, 271V, 272G, 273F

FLR42: E1A, G91A, D96W, E99K, Q249R, G263A, G266S, L269N, 270A, 271G, 272R, 273F

FLR43: E1A, G91A, D96W, E99K, Q249R, L264P, -G266, L269I, 270P, 271R, 272G, 273F

FLR44: E1A, G91A, D96W, E99K, Q249R, L264C, I265N, G266P, T267stop

FLR45: E1A, G91A, D96W, E99K, (R232L), Q249R, G266S, 270A

FLR46: E1A, G91A, D96W, E99K, Q249R, G266S, 270D, 271G

FLR47: E1A, G91A, D96W, E99K, Q249R, L264F, I265D, -G266, 270A, 271G, 272G, 273F

FLR51:E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G

FLR52: E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G

FLR55: E1A, G91A, D96W, E99K, Q249R, G266D

FLR57: E1A, G91A, D96W, E99K, Q249R, G266A, 270P, 271G

FLR58: E1A, G91A, D96W, E99K, Q249R, L264P, I265F, L269stop

FLR59: E1A, G91A, D96W, E99K, Q249R, G266D, L269S, 270A, 271G, 272G, 273F

FLR60: E1A, D27G, G91A, D96W, E99K, Q249R, G266S, L269N, 270A, 271G, 272G, 273F

FLR61: E1A, G91A, D96W, E99K, Q249R, G266D, L269N, 270A

FLR62: E1A, G91A, D96W, E99K, Q249R, L264P, L267Q, L269N

FLR63: E1A, G91A, D96W, E99K, Q249R, G263R, I265L, L269N, 270P

FLR64: E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

FLR66: E1A, G225R, G266D

FLR67: E1A, G225R, G263A, I265V, G266S

FLR68: E1A, G225R, G263A, T267A

FLR73: E1A, G91A, D96W, E99K, Q249R, G266A

HL1032: G266D

G266A

G266S

D96W,S,L, G266A,S,D,C

The above variants with (274S) in parentheses may possibly have a further C-terminal extension of WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNN-QARS (corresponding to the C-terminal of the *F. oxysporum* lipase) in full or truncated form.

Nomenclature for amino acid alterations

The nomenclature used herein for defining mutations is basically as described in WO 92/05249. Thus, G91A indicates substitution of G in position 91 with A. T267A,Q indicates substitution of T at position 267 with A or Q. T267stop indicates a stop codon,

i.e. deletion of T267 and all following amino acids (i.e. C268 and L269). 270P, 271V indicates a C-terminal extension of PV (i.e. at new positions 270 and 271). -G266 indicates deletion of G at position 266. Parentheses indicate that the alteration is optional, or in examples that the alteration is uncertain. SPIRR indicates an N-terminal extension. D266 may refer to the position or to substitution with any amino acid (except D).

Phospholipase activity

The variant of the invention has higher phospholipase activity than the parent lipolytic enzyme. By the monolayer method described later in this specification, the variant preferably has a phospholipase activity of at least 0.1 nmol/min at pH 5.

By the PHLU method described later in this specification, the variant preferably has a phospholipase activity of at least 100 PHLU/mg (mg of pure enzyme protein), particularly at least 500 PHLU/mg. The variant has a ratio of phospholipase activity to lipase activity (both measured at pH 7) of at least 0.1 PHLU/LU, preferably at least 0.5, particularly at least 2.

The variants of the invention have the ability to hydrolyze intact phospholipid, as demonstrated by the PHLU method. They may have A_1 and/or A_2 activity, so they may be able to hydrolyze one or both fatty acyl groups in the phospholipid.

pH optimum

As illustrated in the Examples, some variants of the invention have an alkaline pH optimum for lipase activity and an acid pH optimum for phospholipase activity (e.g. pH 9-10 for lipase and pH 4-6 for phospholipase). Such variants can be used at acid pH (e.g. in baking or oil degumming, described later), as phospholipases with very low concomitant lipase activity.

Thermostability

The thermostability of the variant can conveniently be evaluated by means of Differential Scanning Calorimetry (DSC). Depending on exact mutations, the variants of the invention generally have similar or slightly lower thermostability than the parent lipolytic enzyme.

The temperature at the top of the denaturation peak (T_d) of the lipase from *Humicola laniginosa* when heated at 90 deg/hr at pH 5 is just above 70 °C (=T_d). T_d for the variants of the invention is generally 5-10 degrees lower

Use of variant

The variant of the invention may be used for any purpose where phospholipase activity is desired. Depending on the intended use. the lipase activity can be increased or suppressed by a suitable choice of variant and of pH.

As an example, the variant of the invention can be used in the preparation of dough, bread and cakes, e.g. to improve the elasticity of the bread or cake. Thus, the variant can be used in a process for making bread, comprising adding the variant to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.) or EP 426211 (Unilever). It is particularly advantageous to use an enzyme combination as described in DK 0543/98.

The variant of the invention can also be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the variant. This is particularly applicable to a solution or slurry containing a starch hydrolysate, especially a wheat starch hydrolysate since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

The variant of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the variant so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil. The treatment is preferably carried out at acid pH, e.g. pH 3-5. Advantageously, at acid pH the variant of the invention has a high phospholipase activity and a low lipase activity, due to different pH optima of the two activities.

The process for oil treatment can be conducted according to principles known in the art, e.g. in analogy with US 5,264,367 (Metallgesellschaft, Röhm); K. Dahlke & H. Buchold, INFORM, 6 (12), 1284-91 (1995); H. Buchold, Fat Sci. Technol., 95 (8), 300-304 (1993); JP-A 2-153997 (Showa Sangyo); or EP 654,527 (Metallgesellschaft, Röhm).

The variant of the invention can further be incorporated in a detergent or rinse composition, e.g. for dishwashing as described in GB 2,247,025 (Unilever) or in leather treatment, as described in JP-A 7-177884 (Kao).

The variant of the invention may also be used in the processing of dairy and other food products, e.g. as described in EP 567,662 (Nestlé), EP 426,211 (Unilever), EP 166,284 (Nestlé), JP-A 57-189638 (Yakult) or US 4,119,564 (Unilever).

The variant can be used to prepare lyso-phospholipid (e.g. lyso-lecithin) by treating the corresponding phospholipid with the variant, e.g. as described in EP

870840, JP-A 10-42884, JP-A 4-135456 or JP-A 2-49593. The variant can also be used to make mayonnaise, e.g. as described in EP 628256, EP 398666 or EP 319064.

Methods for preparing enzyme variants

The enzyme variant of the invention can be prepared by methods known in the art, e.g. as described in WO 97/04079 (Novo Nordisk). The following describes methods for the cloning of enzyme-encoding DNA sequences, followed by methods for generating mutations at specific sites within the enzyme-encoding sequence.

Cloning a DNA sequence encoding a enzyme

The DNA sequence encoding a parent enzyme may be isolated from any cell or microorganism producing the enzyme in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the enzyme to be studied. Then, if the amino acid sequence of the enzyme is known, labeled oligonucleotide probes may be synthesized and used to identify enzyme-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide probe containing sequences homologous to another known enzyme gene could be used as a probe to identify enzyme-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying enzyme-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for enzyme (*i.e.* maltose), thereby allowing clones expressing the enzyme to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, *e.g.* the phosphoroamidite method described S.L. Beaucage and M.H. Caruthers, (1981), Tetrahedron Letters 22, p. 1859-1869, or the method described by Matthes et al., (1984), EMBO J. 3, p. 801-805. In the phosphoroamidite method, oligonucleotides are synthesized, *e.g.* in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., (1988), Science 239, 1988, pp. 487-491.

Site-directed mutagenesis

Once a enzyme-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. In a specific method, a single-stranded gap of DNA, the enzyme-encoding sequence, is created in a vector carrying the enzyme gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984), Biotechnology 2, p. 646-639. US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into enzyme-encoding DNA sequences is described in Nelson and Long, (1989), Analytical Biochemistry 180, p. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Further, Sierks. et al., (1989) "Site-directed mutagenesis at the active site Trp120 of *Aspergillus awamori* glucoamylase. Protein Eng., 2, 621-625; Sierks et al., (1990), "Catalytic mechanism of fungal glucoamylase as defined by mutagenesis of Asp176, Glu179 and Glu180 in the enzyme from *Aspergillus awamori*". Protein Eng. vol. 3, 193-198; also describes site-directed mutagenesis in an *Aspergillus* glucoamylase.

Expression of enzyme variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

Expression vector

The recombinant expression vector carrying the DNA sequence encoding a enzyme variant of the invention may be any vector which may conveniently be subjected

to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. The vector may be one which, wh n introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Examples of suitable expression vectors include pMT838.

Promoter

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA sequence encoding a enzyme variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, the TPI (triose phosphate isomerase) promoter from *S. cerevisiae* (Alber et al. (1982), J. Mol. Appl. Genet 1, p. 419-434, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

Expression vector

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* g⁻n⁻s from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistanc . Furth rmore, the vector may comprise *As-*

pergillus selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, *e.g.* as described in WO 91/17243.

The procedures used to ligate the DNA construct of the invention encoding a enzyme variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

Host Cells

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a enzyme variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, *e.g.* by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are Gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, *e.g. Saccharomyces cerevisiae*.

The host cell may also be a filamentous fungus *e.g.* a strain belonging to a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger, or* a strain of *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gribberella zeae,* previously *Sphaeria zeae, synonym with Gibberella roseum and Gibberella roseum* f. sp. *cerealis)*, or *Fusarium sulphureum (*in the prefect

state named *Gibberella puricaris*, synonym with *Fusarium trichothecioides, Fusarium bactridioides, Fusarium sambucium, Fusarium roseum,* and *Fusarium roseum var. graminearum), Fusarium cerealis* (synonym with *Fusarium crokkwellnse), or Fusarium venenatum.*

In a preferred embodiment of the invention the host cell is a protease deficient of protease minus strain.

This may for instance be the protease deficient strain *Aspergillus oryzae* JaL 125 having the alkaline protease gene named "alp" deleted. This strain is described in WO 97/35956 (Novo Nordisk).

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host micro-organism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

Method of producing the enzyme variant of the invention

The enzyme variant of the invention may be produced by a method comprising cultivating a host cell under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the enzyme variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (*e.g.* as described in catalogues of the American Type Culture Collection).

The enzyme variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Expression of variant in plants

The present invention also relates to a transgenic plant, plant part or plant cell which has been transformed with a DNA sequence encoding the variant of the invention so as to express and produce this enzyme in recoverable quantities. The enzyme may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the recombinant enzyme may be used as such.

The transgenic plant can be dicotyledonous or monocotyledonous, for short a dicot or a monocot. Examples of monocot plants are grasses, such as meadow grass (blue grass, Poa), forage grass such as festuca, lolium, temperate grass, such as Agrostis, and cereals, e.g. wheat, oats, rye, barley, rice, sorghum and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous (family Brassicaceae), such as cauliflower, oil seed rape and the closely related model organism Arabidopsis thaliana.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers. In the present context, also specific plant tissues, such as chloroplast, apoplast, mitochondria, vacuole, peroxisomes and cytoplasm are considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part.

Also included within the scope of the invention are the progeny of such plants, plant parts and plant cells.

The transgenic plant or plant cell expressing the variant of the invention may be constructed in accordance with methods known in the art. In short the plant or plant cell is constructed by incorporating one or more expression constructs encoding the enzyme of the invention into the plant host genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

Conveniently, the expression construct is a DNA construct which comprises a gene encoding the enzyme of the invention in operable association with appropriate regulatory sequences required for expression of the gene in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences is determined, eg on the basis of when, where and how the enzyme is desired to be expressed. For instance, the expression of the gene encoding the enzyme of the invention may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are eg described by Tague et al, Plant, Phys., 86, 506, 1988.

For constitutive expression the 35S-CaMV promoter may be used (Franck et al., 1980. Cell 21: 285-294). Organ-specific promoters may eg be a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990. Annu. Rev. Genet. 24: 275-303), or from metabolic sink tissues such as meristems (Ito et al., 1994. Plant Mol. Biol. 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin or albumin promoter from rice (Wu et al., Plant and Cell Physiology

Vol. 39, No. 8 pp. 885-889 (1998)), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* described by Conrad U. et al, Journal of Plant Physiology Vol. 152, No. 6 pp. 708-711 (1998), a promotter from a seed oil body protein (Chen et al., Plant and cell physiology vol. 39, No. 9 pp. 935-941 (1998), the storage protein napA promoter from Brassica napus, or any other seed specific promoter known in the art, eg as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the rbcs promoter from rice or tomato (Kyozuka et al., Plant Physiology Vol. 102, No. 3 pp. 991-1000 (1993), the chlorella virus adenine methyltransferase gene promoter (Mitra, A. and Higgins, DW, Plant Molecular Biology Vol. 26, No. 1 pp. 85-93 (1994), or the aldP gene promoter from rice (Kagaya et al., Molecular and General Genetics Vol. 248, No. 6 pp. 668-674 (1995), or a wound inducible promoter such as the potato pin2 promoter (Xu et al, Plant Molecular Biology Vol. 22, No. 4 pp. 573-588 (1993).

A promoter enhancer element may be used to achieve higher expression of the enzyme in the plant. For instance, the promoter enhancer element may be an intron which is placed between the promoter and the nucleotide sequence encoding the enzyme. For instance, Xu et al. *op cit* disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The DNA construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, micro injection, particle bombardment, biolistic transformation, and electroporation (Gasser et al, Science, 244, 1293; Potrykus, Bio/Techn. 8, 535, 1990; Shimamoto et al, Nature, 338, 274, 1989).

Presently, *Agrobacterium tumefaciens* mediated gene transfer is the method of choice for generating transgenic dicots (for review Hooykas & Schilperoort, 1992. Plant Mol. Biol. 19: 15-38), however it can also be used for transforming monocots, although other transformation methods are generally preferred for these plants. Presently, the method of choice for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992. Plant J. 2: 275-281; Shimamoto, 1994. Curr. Opin. Biotechnol. 5: 158-162; Vasil et al., 1992. Bio/Technology 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh S, et al., Plant Molecular biology Vol. 21, No. 3 pp. 415-428 (1993).

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well-known in the art.

MATERIALS AND METHODS

Lipase activity (LU)

A substrate for lipase is prepared by emulsifying tributyrin (glycerin tributyrate) using gum Arabic as emulsifier. The hydrolysis of tributyrin at 30 °C at pH 7 is followed in a pH-stat titration experiment. One unit of lipase activity (1 LU) equals the amount of enzyme capable of releasing 1 µmol butyric acid/min at the standard conditions.

Phospholipase activity (PHLU)

Phospholipase activity (PHLU) is measured as the release of free fatty acids from lecithin. 50 µl 4% L-alpha-phosphatidylcholine (plant lecithin from Avanti), 4 % Triton X-100, 5 mM $CaCl_2$ in 50 mM HEPES, pH 7 is added 50 µl enzyme solution diluted to an appropriate concentration in 50 mM HEPES, pH 7. The samples are incubated for 10 min at 30 °C and the reaction stopped at 95 °C for 5 min prior to centrifugation (5 min at 7000 rpm). Free fatty acids are determined using the NEFA C kit from Wako Chemicals GmbH; 25 µl reaction mixture is added 250 µl Reagent A and incubated 10 min at 37 °C. Then 500 µl Reagent B is added and the sample is incubated again, 10 min at 37 °C. The absorption at 550 nm is measured using an HP 8452A diode array spectrophotometer. Samples are run in at least in duplicates. Substrate and enzyme blinds (preheated enzyme samples (10 min at 95 °C) + substrate) are included. Oleic acid is used as a fatty acid standard. 1 PHLU equals the amount of enzyme capable of releasing 1 µmol of free fatty acid/min at these conditions.

Phospholipase monolayer assay

On a thoroughly purified surface of a buffer solution (either 10 mM Glycin, pH 9.0 or 10 mM NaOAc, pH 5.0; 1 mM CaCl2, 25°C) a monolayer of Di-Decanoyl-Phosphatidyl Choline (DDPC) is spread from a chloroform solution. After relaxation of the monolayer (evaporation of chlorofom) the surface pressure is adjusted to 15 mN/m, corresponding to a mean molecular area of DDPC of approx. 63 $Å^2$/molec. A solution containing approximately 60 µg (micro gram) enzyme is injected through the monolayer into the subphase of the re-action compartment (cylinder with surface area 2230 mm2 and reaction volume 56570 mm3) in the "zero-order trough". Enzymatic activity is manifested through the speed of a mobile barrier compressing the monolayer in order

to maintain constant surface pressure as insoluble substrate molecules are hydrolysed into more water soluble reaction products. Having verified that the aqueous solubility of the reaction products (capric acid and MDPC) are considerably higher than for DDPC the number of DDPC-molecules hydrolyzed per minute by the enzyme is estimated from the mean molecular area (MMA) of DDPC. The results are calculated on basis of average barrier speed over the first 5 minutes of hydrolysis.

Yeast Strain

Saccharomyces cerevisiae YNG318: MATa leu2-D2 ura3-52 his4-539 pep4-D1[cir+] , described in WO 97/04079 and WO 97/07205.

Transformation of yeast strain

The DNA fragments and the opened vectors are mixed and transformed into the yeast *Saccharomyces cerevisiae* YNG318 by standard methods.

Vector for yeast transformation

pJSO026 (*S. cerevisiae* expression plasmid) is described in WO 97/07205 and in J.S.Okkels, (1996) "A URA3-promoter deletion in a pYES vector increases the expression level of a fungal lipase in Saccharomyces cerevisiae. Recombinant DNA Biotechnology III: The Integration of Biological and Engineering Sciences, vol. 782 of the Annals of the New York Academy of Sciences). It is derived from pYES 2.0 by replacing the inducible GAL1-promoter of pYES 2.0 with the constitutively expressed TPI (triose phosphate isomerase)-promoter from *Saccharomyces cerevisiae* (Albert and Karwasaki, (1982), J. Mol. Appl Genet., 1, 419-434), and deleting a part of the URA3 promoter.

Screening method

The yeast libraries are spread on cellulose filters on SC-ura agar plates and incubated for 3-4 days at 30°C.

The filters are then transferred to the lecithin plates and incubated at 37°C for 2-6 hours. Yeast cells harbouring active phospholipases will develope white clearing zones around the colonies. The positive variants can then be further purified and tested.

Media

<u>SC-ura medium</u>

Yeast Nitrogen (without amino aicds)	7.5 g

Succinic acid	11.3 g
NaOH	6.8 g
Casaminoacid (without vitamins)	5.6 g
Tryptophan	0.1 g
Agar, Merck	20 g
Distilled water	ad 1000 ml

Autoclaved for 20 minutes at 121°C.

From a sterile stock solution of 5% Threonine 4 ml is added to a volume of 900 ml together with 100 ml of a sterile 20% glucose.

Preparation of Lecithin plates:

10 g agarose is melted in 550 ml H_2O by boiling in a microwave oven. After cooling to 60-70°C the following ingredients are added:

250 ml of a 0.4 M Citrate buffer (pH 4.5 or pH 7.1)

200 ml 3% lecithin (from Avanti) in 2% Triton-X 100

2 ml 2% crystal violet

30 ml of the mixture is poured into 14 cm Ø petri dishes.

EXAMPLES

Example 1

Construction of variants with Lipolase "backbone" and C-terminal from *Fusarium oxysporum* phospholipase by PCR reaction

For the Lipolase backbone the following variants were used as template: HLR908 (E1A, G91A, D96W, E99K, Q249R) or HLR739 (SPIRR, G91A, D96W, E99K, Q249R), HLR907(wt Lipolase) was used for generating a fragment in the C-terminal without Q249R. The template for the C-terminal phospholipase was the F.o. phospholipase, cloned in the same vector as the Lipolase variants.

PCR reaction 1: 4244 as 5' primer and H7 as 3'primer and either HLR908 (to get E1A) or HLR739 (to get SPIRR) was used as template.

PCR reaction 2: FOL14 as 5' primer and FOL15 as 3' primer and HLR907 as template (no mutation in pos 249)

PCR reaction 3: FOL16 as 5' primer and AP as 3' primer and F.o. phospholipase as template

A PCR reaction 4 was made to create the connection between the Lipolase variant and the C-terminal from the phospholipase by using FOL14 as 5' primer and AP as 3' primer and PCR reaction 2 and 3 as template.

The final PCR was made with 4244 as 5' primer and KBoj14 as 3' primer and PCR re-action 1 and 4 as template. (By using HLR907 as template in reaction 2 a posibility to omit the mutation in position 249 was created).

The final PCR fragment was used in an in vivo recombination in yeast together with pJSO026 cut with the restriction enzymes. SmaI(or BamHI) and XbaI (to remove the coding region and at the same time create an overlap of about 75 bp in each end to make a recombination event possible). This final treatment was also used in the following examples.

Primer FOL14 and primer 15/16 are mixed oligoes to give the possibility to bind both with Lipolase and phospholipase templates and at the same time give possibilities for introducing the amino acids from both templates in the different positions. For some of the positions new amino acids could be introduced as well.

Primer FOL14

Position 205 in the *H. lanuginosa* lipase: 75% R, 25% S

Primer FOL15/16

Position 256 in the *H. lanuginosa* lipase: 50% P, 50% A

Position 260 in the *H. lanuginosa* lipase: 25% R, 12.5% Q, 12.5% H, 12.5% C, 12.5% Y, 12.5% W, 12.5% stop.

The resulting variants were denoted FLR2, FLR3, FLR4, FLR5, FLR6 and FLR7. Their sequences were determined as described earlier in the description.

Example 2

Production of truncated sequences

Variants of FLR2 were made with stop after amino acid 269, 270, 271, 272,(273 and 274)

The following PCR reactions were made with FLR 2 as template:

Reaction 1: 5' primer 4244 and 3' primer KBoj36 (stop after 269)

Reaction 2: 5' primer 4244 and 3' primer KBoj37 (stop after 270)

Reaction 3: 5' primer 4244 and 3' primer KBoj38 (stop after 271)

Reaction 4: 5' primer 4244 and 3' primer KBoj39 (stop after 272)

The resulting variants were denoted FLR37, FLR39, FLR51 and FLR52. Their sequences were determined as described earlier in the description.

Example 3

Removal of mutations in the lid region

G91A or E99K can be removed without loosing the phospholipase activity.

The resulting variants were denoted FLR29, FLR30, FLR31 and FLR64. Their sequences were determined as described earlier in the description.

Example 4

Doping in the C-terminal regi n of Lipolase to introduce phosph lipase activity

Three different libraries were constructed with possibilities for mutations in position 256 and position 263-269. At the same time possibilities for extension of the C-terminal with either 1, 2 , 3 or 4 amino acids were included.

Doping, the wt sequences are underlined:

256: P 94, A 3, T 3

263: G 87, E 4.8, A 3.8, R 3.6, Q 0.2, P 0.2

264: L 87, P 4.8, Q 3.8, V 3.6, A 0.2, E 0.2

265: I 85, T 5.6, L 2.2, S 1.6, N 1.5, F 1.4, R 0.4, K 0.4 A,P 0.1, G,D,C,H,Y 0.03, Q,E 0.01, stop 0.016

266: G 86, D 5.9, R 2, S 1.7, C 1.6, A 0.9, V 0.9, E 0.7, W 0.2, H,Y 0.1, I,L,T,F,P 0.02, Q,K 0.01, stop 0.014

267: T 86, A 6.6, S 1.9, R 0.9, N 0.9, I 0.9, K 0.9, M 0.9, P 0.9, P 0.9, G,V 0.14, D,E 0.07, L 0.03, C,Q,H,F,W,Y 0.01, stop 0.01

268: C 91, S 1.9, R 1.0, G 1.0, F 0.9, Y 0.9, L 0.04, A,N,D,H,I,P,T,V 0.01, stop 2.8

269: L 92, stop 8 (KBoj 32 and KBoj33)/ N 86, K 2.7, D 1.8, H 1.8, I 1.8, S 1.8, T 1.9, Y 1.8, R 0.1, Q,M,E 0.06, A,C,G,L,F,P,V 0.04, stop 0.06(KBoj34)

270: stop 100 (KBoj33)/A 44, P 44, S 1.9, T 1.8, R 1.5, L 1.5, G 1.4, V 1.4, D 0.7, Q 0.7, E 0.7, H 0.7, N,C,I,K,M,F,W,Y 0.03, stop 0.03 (KBoj 32 and KBoj 34)

271: G 72, R 4.5, V 3.2, E 3.0, C 2.9, A 1.6, S 1.2, D 1.0, L 0.5, I,K,Y 0.15, Q,T 0.08, N,P 0.05, stop 9.2

272: G 72, R 4.5, V 3.2, E 3.0, C 2.9, A 1.6, S 1.2, D 1.0, L 0.5, I,K,Y 0.15, Q,T 0.08, N,P 0.05, stop 9.2

273: F 74, L 11, S 2.8, I 2.7, V 2.7, Y 2.5, C 2.5, A,R,T 0.1, N,D,H 0.08, Q,E,K 0.01, stop 0.5

274 STOP

Library A: PCR reaction with 4244 as 5' primer and KBoj 33 as 3' primer and HLR908 or HLR1178 (E1A, G225R) as template. Variants from this library will be without extension.

Library B: PCR reaction with 4244 as 5' primer and KBoj 32 as 3' primer and HLR908 or HLR1178 (E1A, G225R) as template. Variants from this library will most probably contain a C-terminal extension but can contain stop codons before the extension.

Library C: PCR reaction with 4244 as 5' primer and KBoj 34 as 3' primer and HLR908 or HLR1178 (E1A, G225R) as template. Variants from this library will most

probably contain mutations in position 269 and a C-terminal extension but can contain stop codons before the extension.

The following variants were obtained (see description earlier in this specification):

Library A: FLR55

Library B: FLR45, FLR46, FLR48, FLR57, FLR58

Library C: FLR41, FLR42, FLR43, FLR59, FLR60, FLR61, FLR62, FLR63.

Example 5

Lipase and phospholipase activities of variants

A number of variants of the invention (described above) were purified and analyzed as follows. A prior-art enzyme from *F. oxysporum* known to have both lipase and phospholipase activity was included for comparison. The results are shown in the table below.

The lipase (LU) and phospholipase (PHLU) activity was measured and expressed as activity per mg of pure enzyme protein (measured by absorption at nm). The ratio of phospholipase to lipase is also shown in the table. Further, the pH optimum was determined by using the LU and PHLU methods at various pH values.

	Variant	Activity		Ratio	pH optimum	
		LU/A_{280}	$PHLU/A_{280}$	PHLU / LU	LU	PHLU
Invention	FLR2	24	173	7.2	9-10	6.0
	FLR6	194	824	4.2	9-10	4 .0
	FLR29	138	424	3.07		4.0
	FLR37	27	229	8.48		4.0
	FLR46	1568				4
	FLR55				6	
	FLR57				10	
	HL1032				6	5
Prior art	*F. oxysporum* wild type	2500	2500	1.0	9-10	9-10

Example 6

Phospholipase activity by monolayer assay

A number of variants were analyzed for phospholipase activity by the mono lay r assay described above at pH 5 and 9. Some prior-art lipases and variants were

included as reference. The results (in nmol/min) were as follows (FoL refers to the *Fusarium oxysporum* lipase/phospholipase):

		pH 5	pH 9
Invention	HL 1032	0.20	0.3
	FLR 2	4.5	3.4
	FLR 6	4.8	6.3
	FLR 29	6.2	7.5
	FLR 37	5.8	5.3
	FLR 46	1.3	2.6
	FLR 55	3.3	0.1
	FLR 57	0.5	0.2
Prior art	Lipolase	0.0	0.0
	Lipolase variant HLR282: SPIRR+N94K+F95L+D96H+N101S+F181 L+D234Y+I252L+P256T+G263A+L264Q	0.0	
	FoL	3.6	5.2

Example 7

Thermostability of variants

The thermostability of variants was tested by DSC (Differential Scanning Calorimetry) by heating at 90 deg/hr at pH 5 (50 mM acetate buffer), pH 9 (50 mM borate buffer) or pH 10 (50 mM glycine buffer). The temperature at the top of the denaturation peak (T_d) was found as follows:

	Variant	pH 5	pH 9	pH 10
Invention	FLR2	54°C	62°C	
	FLR6	57°C	57°C	56°C
	FLR29	58°C	68°C	68°C
	FLR46		64°C	61°C
	HL1032	69°C	65°C	67°C
Prior art	*Fusarium oxysporum* lipase		49°C	

Exampl 8

D gumming of vegetable oil

Rape seed oil was treated with two variants of the invention, essentially as described in Example 6 of WO 98/18912 (Novo Nordisk).

The variant FLR2 was tested at various pH and temperatures. The enzyme dosage was 0.6 mg of enzyme protein per kg of oil. Results:

Temp.	pH	Initial performance	Final P content after 6 hours
45 °C	4.5	0 %	42
45 °C	5.0	40 %	11
45 °C	5.7	100 %	4
45 °C	6.5	60 %	3
35 °C	5.8	90 %	4
50 °C	5.5	0 %	39
55 °C	5.5	0 %	38

The results show optimum performance at pH 5.7, 35-45°C. A separate experiment at 45°C, pH 6 showed that a final P content of 4 ppm could be reached at an enzyme dosage as low as 0.15 mg/kg.

A similar experiment with variant FLR6 showed optimum performance at 40°C, pH 5.0-5.5. The enzyme dosage was 0.3 mg/kg.

A degumming experiment was made with variant HL1032, using rape seed oil at 45°C, pH 5, 1.8 mg enzyme/kg oil. For comparison, a similar experiment was made with the parent lipase (Lipolase) at 18 mg/kg. The results (P content) were as follows:

	Invention	Reference
	HL1032	Lipolase
0 hours	166 ppm	231 ppm
3.4 hours	7 ppm	203 ppm

The results show that good degumming (<10 ppm residual P content) was obtained in 3.4 hours with variant HL1032. The parent lipase had very little degumming effect, even at 10 times higher enzyme dosage.

Example 9

Baking tests

Th⁻ variant FLR-2 was evaluated in baking tests as follows.

Doughs were prepared from Meneba flour according to the European straight dough method (ABF-SP-1201.01). The variant, ascorbic acid, phospholipase (lecithin) and endo-amylase were added to the dough as described below. The endo-amylase was maltogenic amylase from *B. stearothermophilus* (tradename Novamyl(R)). The amylase dosage is given in MANU units. One MANU (Maltogenic Amylase Novo Unit) may be defined as the amount of enzyme required to release one µmol of maltose per minute at a concentration of 10 mg of maltotriose substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37 °C for 30 minutes.

Dough	Ascorbic acid	FLR2 mg enzyme/kg	Phospholipase mg enzyme/kg	Lecithin g/ kg	Novamyl MANU/kg
1	40 ppm	0	0	0	0
2	40 ppm	0	0,4	10	0
3	40 ppm	0,25	0	10	0
4	40 ppm	0,5	0	10	0
5	40 ppm	1,5	0	10	0
6	40 ppm	0	0	10	750
7	40 ppm	1,5	0	10	750
8	40 ppm	0,25	0	10	750

After baking, the loaves were cooled, and the loaf volume, crumb firmness and softness were evaluated after approximately 2 hours. The evaluation was repeated after 1, 3 and 7 days storage at 22°C wrapped in double plastic bags.

Firmness of crumb was measured using a texture analyzer TA-XT2 from Stable Micro Systems (probe diameter 40 mm).

Softness in gram was measured as the force needed to press a probe 6,25 mm into a crumb of a 25 mm thick slice of bread (25 % penetration).

A comparison of loaf volumes for dough 6 and 7 showed that the addition of 1.5 mg of FLR-2 variant increased the loaf volume by 9 %.

The results for firmness and elasticity are shown in Figs. 1 and 2. A comparison of doughs 6, 7 and 8 shows that the FLR-2 variant gives significantly softer crumb and significantly better elasticity from day 0 to day 7.

CLAIMS

1. A variant of a parent lipolytic enzyme, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue, at a position which is within 10 amino acid positions from the C-terminal, and

b) has a higher phospholipase activity than the parent lipolytic enzyme.

2. A variant of a parent lipolytic enzyme having a lid and an alcohol binding site, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue, at a position which is

i) within 10 amino acid positions from the C-terminal, or

ii) no more than 10 Å (preferably no more than 8 Å) from the C atom at the sn2 position of the glycerol part of a substrate triglyceride, or

iii) in the lipolytic enzyme lid, and

b) has a higher phospholipase activity than the parent lipolytic enzyme.

3. The variant of either preceding claim which has a phospholipase activity greater than 0.1 nmol/min in a monolayer assay at pH 5 as described herein.

4. The variant of any preceding claim which has a phospholipase activity greater than 100 PHLU/mg (preferably greater than 500 PHLU/mg) and/or a ratio of phospholipase activity to lipase activity greater than 0.1 PHLU/LU (preferably greater than 0.5 PHLU/LU).

5. The variant of any preceding claim wherein the parent lipolytic enzyme has a phospholipase activity below 50 PHLU/mg and/or a ratio of phospholipase activity to lipase activity below 0.1 PHLU/LU.

6. The variant of any preceding claim which is native to an eukaryote, preferably to a fungus.

7. The variant of any preceding claim wherein the parent lipolytic enzyme belongs to the *Zygomycete* family.

8. The variant of any of claims 1-6 wherein the parent lipolytic enzyme belongs to the *Humicola* family.

9. The variant of the preceding claim wherein the parent lipolytic enzyme is the lipase of *Humicola lanuginosa* strain DSM 4109.

10. The variant of any preceding claim which comprises an alteration which is a substitution at a position corresponding to G266 in the *Humicola lanuginosa* lipase, preferably a substitution G266A, C, D, N, L, I, S, T, P or V.

11. The variant of any preceding claim which comprises an alteration at a position corresponding to position G263, L264, I265, T267 or L269 in the *Humicola lanuginosa* lipase, preferably a substitution which is G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; T267A,Q or L269N.

12. The variant of any preceding claim which comprises an alteration in the lid which is a substitution of a negatively charged amino acid residue with a neutral or positively charged amino acid residue, or a substitution of a neutral amino acid residue with a positively charged amino residue.

13. The variant of the preceding claim which comprises an alteration in the lid at a position corresponding to position G91, D96 and/or E99 in the *Humicola lanuginosa* lipase, preferably a substitution which is G91A, D96S,W or E99K.

14. The variant of any of claims 8-10 wherein the alteration in the alcohol binding site comprises deletion of amino acid residues at positions corresponding to positions C268 and L269 in the lipase derived from *Humicola lanuginosa* strain DSM 4109.

15. The variant of any preceding claim which comprises a peptide extension at the C-terminal, preferably comprising 1-5 amino acid residues, the first preferably being A, P or D, the second (if present) preferably being V, G or R, the third (if present) preferably being V, G or R, the fourth (if present) preferably being F, and the fifth (if present) preferably being S.

16. A DNA sequence encoding the variant of any preceding claim.

17. A vector comprising the DNA sequence of the preceding claim.

18. A transformed host cell harboring the DNA sequence of claim 16 or the vector of claim 17.

19. A method of producing the variant of any of claims 1-15 comprising

a) cultivating the cell of claim 18 so as to express and preferably secrete the variant, and

b) recovering the variant.

20. A method of producing a phospholipase, which method comprises:

a) selecting a parent lipolytic enzyme,

b) identifying one or more amino acid residues in the parent lipolytic enzyme at positions which are within 10 amino acid positions from the C-terminal

c) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps b-d,

f) testing the phospholipase activity of the variant,

g) optionally repeating steps b-f,

h) selecting a variant having higher phospholipase activity than the parent lipolytic enzyme, and

i) producing the variant to obtain the phospholipase.

21. A method of producing a phospholipase, which method comprises:

a) selecting a parent lipolytic enzyme having a lid and an alcohol binding site,

b) identifying one or more amino acid residues in the parent lipolytic enzyme at positions which are:

i) within 10 amino acid positions from the C-terminal, or

ii) no more than 10 Å from the C atom at the sn2 position of the glycerol part of a substrate triglyceride, or

iii) in the lipolytic enzyme lid, and

c) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps b-d,

f) testing the phospholipase activity of the variant,

g) optionally repeating steps b-f,

h) selecting a variant having higher phospholipase activity than the parent lipolytic enzyme, and

i) producing the variant to obtain the phospholipase.

22. A process for preparing a dough or a baked product prepared from the dough which comprises adding the variant of any of claims 1-15 to the dough.

23. The process of the preceding claim which further comprises adding to the dough an endo-amylase and a phospholipid.

24. The process of either preceding claim wherein the endo-amylase is from *Bacillus*, and is preferably a maltogenic amylase from *B. stearothermophilus*.

25. A process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the variant of any of claims 1-15 so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

Primers

4244: 5'-TCA AGA ATA GTT CAA ACA AGA AGA-3'

AP: 5'-GGT TGT CTA ACT CCT TCC TTT TCG-3'

FOL 14: 5'-TGT CCC YMG WCT CCC KCC K-3'

FOL 15: 5'-GAA GTA MYR YAG RTG MGC AGS RAT ATC-3'

FOL16: 5'-GAT ATY SCT GCK CAY CTR YRK TAC TTC-3'

H7: 5'-CGG AAT GTT AGG CTG GTT ATT GC-3'

KBoj 14: 5'-CTT TTC GGT TAG AGC GGA TG-3'

KBoj 32: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA 122 345 345 S67 C8A 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj 33: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CGC CGC GCA CTA C8A 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj34: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA 122 345 345 S67 201818 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj36: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA GTT ACA GGC GTC AGT CGC CTG GAA G

KBoj37: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA AGC GTT ACA GGC GTC AGT CGC CTG G

KBoj38: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA ACC AGC GTT ACA GGC GTC AGT CGC C

KBoj39: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA GCC ACC AGC GTT ACA GGC GTC AGT C

Distribution of nucleotides for each doped position

1: A 90, C 10

2: G 3,A 91,T 3,C 3

3: A 25, T 75

4: G 2, A 4, T 5, C 89

5: G 2, A 13, T 4, C 81

6: G 91, A 3, T 3, C 3

7: G 48, A 2, T 2, C 48

8: A 92, T8

9: A 97, T 3

10: G 1, A 1, T 1, C 97

11: G 1, A 97, T 1, C 1
12: G 94, A 2, T 2, C 2
13: G 1, A 1, T 91, C 7
14: G 1, A 1, T 7, C 91
15: G 2, A 2, T 2, C 94
16: A 80, T 20
17: G 6, A 90, T 2, C 2
18: G 2, A 2, T 94, C 2
19: G 5, A 91, T 4
20: G 96, C4
21: G 4, T 5, C 91
22: G 4, C 96
23: G 94, C 3, T 3

```
          1                                                  50
  rhimi   SIDGGIRAAT SQEINELTYY TTLSANSYCR TVIPGA...T WDC..IHCDA
  rhidl   SDGGKVVAAT TAQIQEFTKY AGIAATAYCR SVVPGN...K WDC..VQCQK
  SP400   ~~~~~~~~EVS QDLFNQFNLF AQYSAAAYCG KNNDAPAGTN ITCTGNACPE
    Pcl   ~~~~~~~~DVS TSELDQFEFW VQYAAASYYE ADYTAQVGDK LSCSKGNCPE
FoLnp11   ~~~~~~~~GVT TTDFSNFKFY IQHGAAAYC. .NSEAAAGSK ITCSNNGCPT

          51                                                 100
  rhimi   TE..DLKIIK TWS.TLIYDT NAMVARGDSE KTIYIVFRGS SSIRNWIADL
  rhidl   WV.PDGKIIT TFT.SLLSDT NGYVLRSDKQ KTIYLVFRGT NSFRSAITDI
  SP400   VEKADATFLY SFEDSGVGDV TGFLALDNTN KLIVLSFRGS RSIENWIGNL
    Pcl   VEATGATVSY DFSDSTITDT AGYIAVDHTN SAVVLAFRGS YSVRNWVADA
FoLnp11   VQGNGATIVT SFVG.SKTGI GGYVATDSAR KEIVVSFRGS INIRNWLTNL

          101                                                150
  rhimi   TFVPVSY.PP VSGTKVHKGF LDSYGEVQNE LVATVLDQFK QYPSYKVAVT
  rhidl   VFNFSDY.KP VKGAKVHAGF LSSYEQVVND YFPVVQEQLT AHPTYKVIVT
  SP400   NFDLKEINDI CSGCRGHDGF TSSWRSVADT LRQKVEDAVR EHPDYRVVFT
    Pcl   TFVHTNP.GL CDGCLAELGF WSSWKLVRDD IIKELKEVVA QNPNYELVVV
FoLnp11   DFGQEDC.SL VSGCGVHSGF QRAWNEISSQ ATAAVASARK ANPSFNVIST

          151                                                200
  rhimi   GHSLGGATAL LCALDLYQRE EGLSSSNLFL YTQGQPRVGD PAFANYVVST
  rhidl   GHSLGGAQAL LAGMDLYQRE PRLSPKNLSI FTVGGPRVGN PTFAYYVEST
  SP400   GHSLGGALAT VAGADLRGN. .GY...DIDV FSYGAPRVGN RAFAEFLTVQ
    Pcl   GHSLGAAVAT LAATDLRGK. .GYP..SAKL YAYASPRVGN AALAKYITAQ
FoLnp11   GHSLGGAVAV LAAANLRVG. .GT...PVDI YTYGSPRVGN AQLSAFVSNQ

          201                                                250
  rhimi   G.IPYRRTVN ERDIVPHLPP AAFGFLHAGE EYWITD.NSP ......ETVQ
  rhidl   G.IPFQRTVH KRDIVPHVPP QSFGFLHPGV ESWIKS.GT. ......SNVQ
  SP400   TGGTLYRITH TNDIVPRLPP REFGYSHSSP EYWIKS.GTL V.PVTRNDIV
    Pcl   G..NNFRFTH TNDPVPKLPL LSMGYVHVSP EYWITS.PNN A.TVSTSDIK
FoLnp11   A.GGEYRVTH ADDPVPRLPP LIFGYRHTTP EFWLSGGGGD KVDYTISDVK

          251                                                300
  rhimi   VCTSDLETSD CSNSIVPFT. .SVLDHLSYF GINTGLCT~~ ~~~~~~~~~~
  rhidl   ICTSEIETKD CSNSIVPFT. .SILDHLSYF DINEGSCL~~ ~~~~~~~~~~
  SP400   KIEGID.ATG GNNQP.NIP. .DIPAHLWYF .GLIGTCL~~ ~~~~~~~~~~
    Pcl   VIDGDV.SFD GNTGTGLPLL TDFEAHIWYF .VQVDAGKGP GLPFKRV~~~
FoLnp11   VCEGAA.NLG CNGGT.LGL. .DIAAHLHYF .QATDACNAG GFSWRRYRSA

          301                                          338
  rhimi   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~...
  rhidl   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~...
  SP400   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~...
    Pcl   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~...
FoLnp11   ESVDKRATMT DAELEKKLNS YVQMDKEYVK NNQARS..
```

Fig. 1

Alignment of lipase sequences

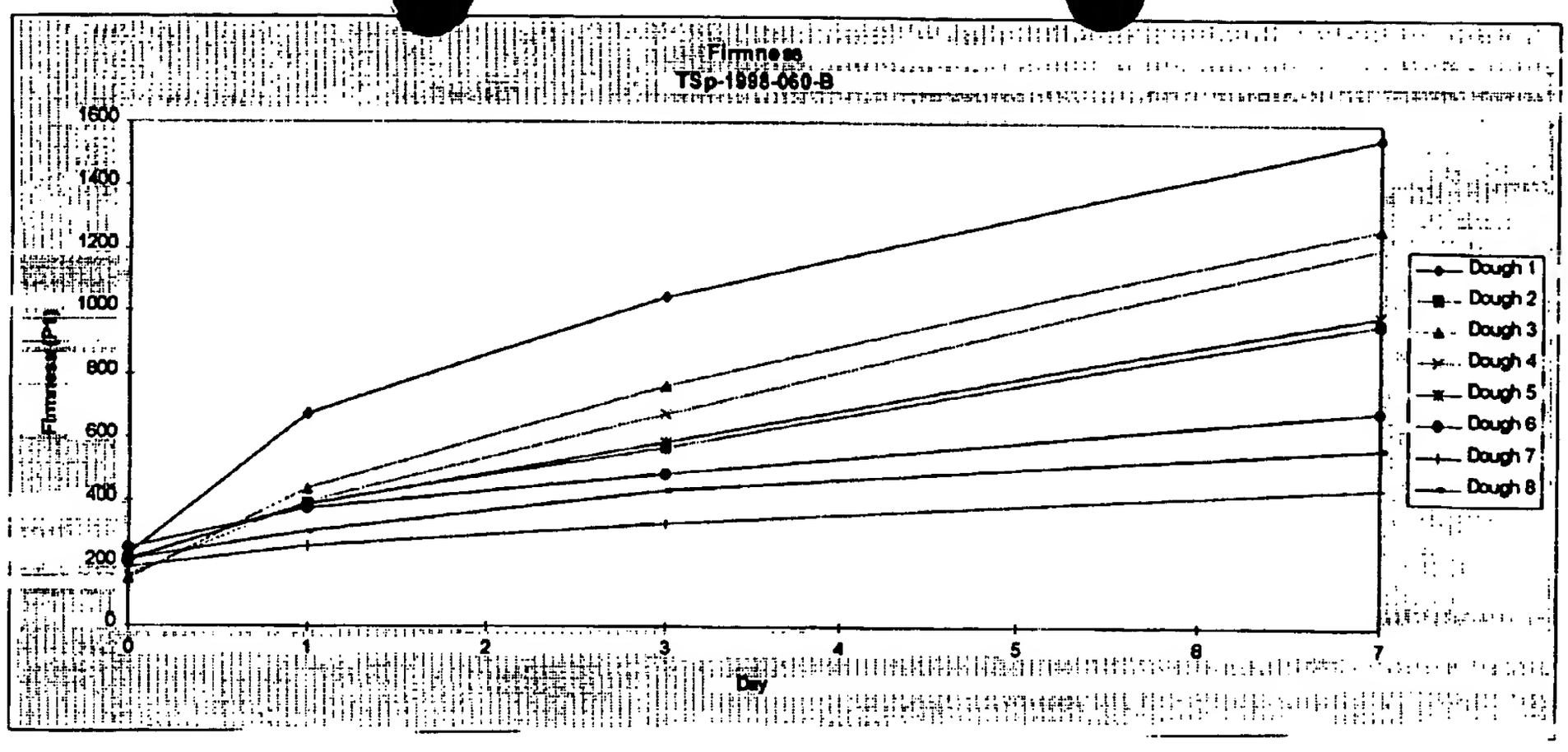

Fig. 2

Firmness of bread

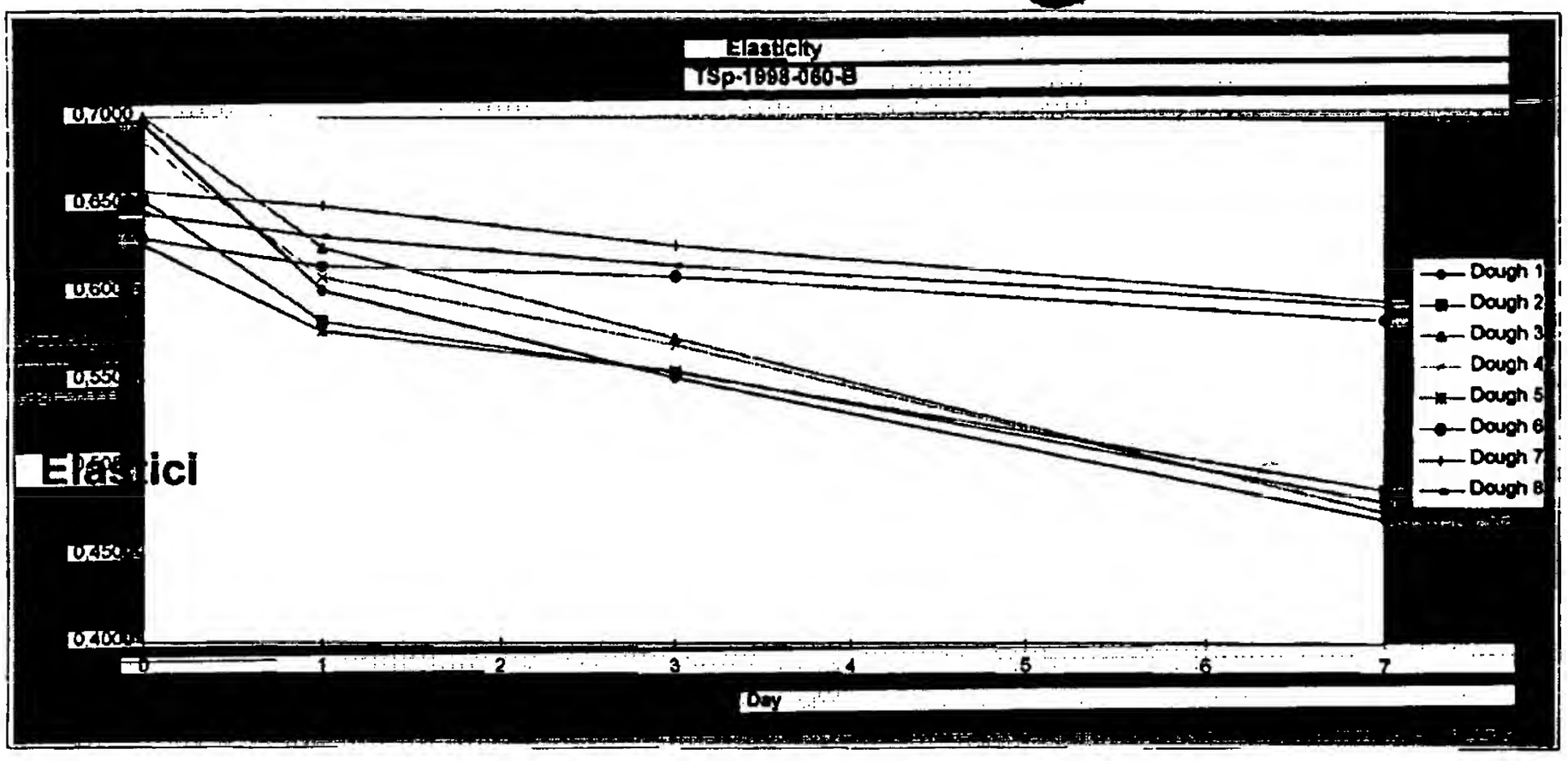

Fig. 3

Elasticity of bread